How to Detect Weak Pairs

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Many biological processes involve nonsynchronized, stochastic changes in conformation and in molecular interactions. A fundamental understanding of these processes requires direct, real-time observation on the molecular scale. Single-molecule fluorescence is emerging as a general, minimally invasive tool for the in vitro and in vivo study of such systems, including enzyme dynamics (*1, 2*), protein folding (*3, 4*), and ribozyme folding (*5*).

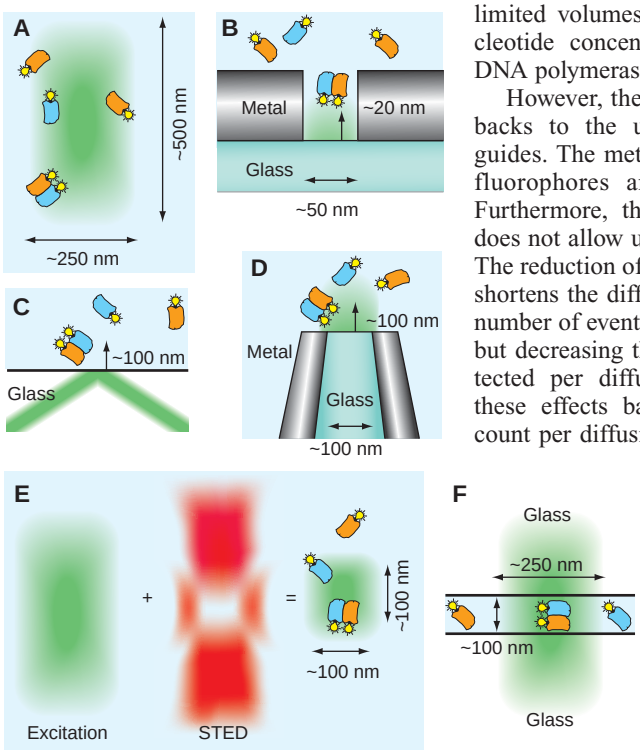
On page 682 of this issue, Levene *et al.* (*6*) report a method that enables the single-molecule detection of even weaker, transient interactions between proteins, between proteins and nucleic acids, and between enzymes and substrates. By circumventing the diffraction limit of light, they can detect individual molecules at much higher (micromolar) concentrations than previously possible.

Single-molecule detection requires sufficient signal intensity from a molecule to overcome intrinsic noise, and the ability to capture one molecule's signal while excluding signals from other molecules and the background. Fluorescence signals from single molecules reach 10 to 100 kHz, and the red shift of the emission spectrum with respect to the excitation spectrum allows discrimination from background sources. But light diffraction produces an inherent resolution limit (~250 nm for visible light), setting a minimum detection volume of ~0.2 femtoliters (1 fl = 10^{-15} l).

This limit applies to confocal microscopy, which selects the volume with a tightly focused laser excitation and a detection pinhole (panel A in the figure). To isolate single molecules, the sample must be dilute so that on average less than one molecule resides in the detection volume. The concentration must not exceed 100 pM to 1 nM, limiting solution-based single-molecule fluorescence studies of interacting molecules to relatively strong interactions.

Consider two labeled species A and B, with concentrations [A] and [B], that interact (A + B ⇄ AB) with a dissociation constant K_d = [A][B]/[AB]. The brightness of AB is (ideally) double that of A or B, and the diffusion time of AB is longer than that of A or B. Changes in brightness and diffusion times thus indicate interactions. But observations of single-molecule interactions in femtoliter detection volumes are impractical for dissociation constants in the micromolar range. For example, if K_d = 1 μM and [A] = [B] = 1 nM, then [AB] = 1 pM, 1000 times lower than [A] or [B], which therefore mask AB.

To study interactions with micromolar dissociation constants, it is necessary to reduce the detection volume with subdiffraction (superresolution) optical methods.



Detecting single molecules in reduced volumes. Molecules A and B are both labeled with fluorophores (yellow bulbs). (**A**) In confocal microscopy, the detection volume is limited by diffraction. (**B**) Zero-mode waveguides use wide-field illumination; small holes prevent light propagation but allow evanescent fields. (**C**) In TIR-FCS, a large angle of incidence excludes light propagation but allows evanescent fields. (**D**) In NSOM, a tapered fiber tip prevents light propagation but allows evanescent fields. (**E**) STED uses excitation beam and detection pinhole, as in confocal microscopy; an additional STED beam carves away the wings of the excitation profile. (**F**) In nanofluidic channels, the movement of molecules is physically restricted to regions smaller than the diffraction limit.

The superresolution method of Levene *et al.* (*6*) allows detection volumes as small as 10 zeptoliters (1 zl = 10^{-21} l). The volume can be matched to the concentrations used (100 nM to 200 μM). A thin metal film is deposited on fused silica coverslips, and holes with diameters of 30 to 80 nm are etched in the film. The holes act as waveguides that are too small to sustain propagating light modes (zero-mode waveguides). Evanescent waves that decay within ~20 nm inside the waveguide are used to excite fluorescence (panel B).

The authors demonstrate single-molecule detection and fluorescence correlation spectroscopy (FCS) (*7*) by monitoring the incorporation of fluorescent nucleotides by DNA polymerase. Incorporation events are seen as photon bursts, which end when the incorporated fluorescent nucleotides photobleach (that is, stop to fluoresce). These events cannot be observed in diffraction-limited volumes because micromolar nucleotide concentrations are required for DNA polymerase function.

However, there are also possible drawbacks to the use of zero-mode waveguides. The metal surfaces may affect the fluorophores and macromolecules (*8*). Furthermore, the experimental geometry does not allow use of this method in cells. The reduction of the detection volume also shortens the diffusion time, increasing the number of events measurable per unit time but decreasing the number of photons detected per diffusing molecule. In FCS, these effects balance out if the photon count per diffusion time is ≥1 (*7*), but the decreased photon count may reduce the sensitivity of photon-counting histogram methods and single-molecule burst methods (*7*).

Levene *et al.* provide the first demonstration of high-concentration (micromolar) single-molecule spectroscopy. Their method allows parallel observation of many detection volumes, each volume smaller than with previous methods. Other superresolution methods include total internal reflection FCS (TIR-FCS, panel C) (*9*), which can reduce the detection volume

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to ~20 attoliters (1 al = 10^{-18} l), and near-field scanning optical microscopy (NSOM, panel D) (*10*), which allows attoliter detection volumes. NSOM is best suited to cell-surface studies where scanning is important.

Single-molecule detection in reduced detection volumes can also be achieved with stimulated emission depletion (STED, panel E) (*11, 12*). With this method, detection volumes have been reduced to 0.67 attoliters (*11*). The detection volume can be placed anywhere in solution, including inside a cell. However, use of STED with more than one color of fluorophore is difficult, and the STED beam is of high intensity.

Another possibility is the use of nanofluidic channels to restrict the movement of molecules to a width and depth smaller than the confocal detection volume (panel F) (*13*). Molecules would not be near metal surfaces, and diffusion times would be longer because diffusion would be effectively one-dimensional. Finally, if super-resolution lenses can be made from materials with a negative index of refraction, they may in the future be used for single-molecule detection in small volumes (*14*).

All these methods have strengths in different areas and are suitable for different applications. The strength of the zero-mode waveguide of Levene *et al.* (*6*) is that it allows parallel detection of many single molecules while providing the smallest detection volumes of any method reported to date, enabling single-molecule analysis of much weaker interactions than previously possible.

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COMPUTER SCIENCE

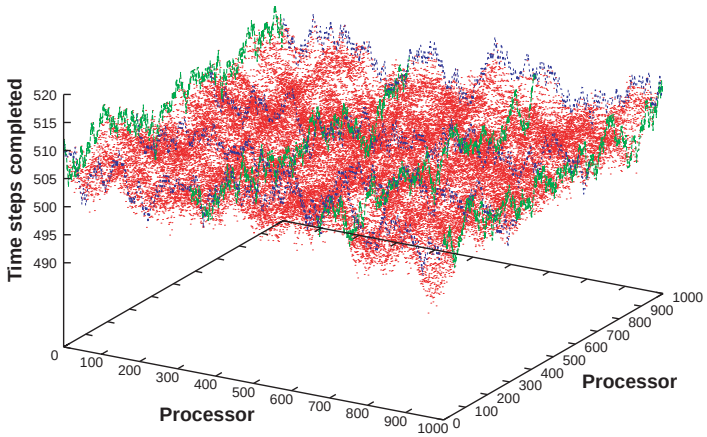
Rough Times Ahead

Scott Kirkpatrick

High-performance computing has moved from being a problem of optimizing the architecture of an individual supercomputer to one of optimizing the organization of large numbers of ordinary computers operating in parallel (*1*). This development has been made possible by rapid progress in microprocessor, memory, and storage components.

On page 677 of this issue, Korniss *et al.* (*2*) point out a key weakness of this approach. Simulated time, the common parameter linking the many loosely coupled elements of a distributed simulation, can get rough. Just as the surfaces of crystalline materials grown by depositing individual atoms roughen (*3*), so does the temporal surface of a computation spread continuously as the modeled system evolves.

This roughening becomes a problem when measurements must be continuously extracted from the complex simulation. The solution proposed by Korniss *et al.* takes advantage of a property found in the Internet: its "small world" nature of having occasional links between points that would otherwise be thought to be far apart. The authors show that intermittent synchronization over random distances can suppress temporal roughening.



Out of sync. This simulated temporal surface was created by allowing 1000 x 1000 processing elements to proceed in equal time steps under local synchronization.

The best evidence of the changing nature of high-performance computing comes from the regularly updated "TOP500" survey of the world's 500 biggest computing complexes (*4*). By 2002, clusters of conventional computers had come to make up 93% of the listed machines, with old-fashioned, vector-based supercomputers constituting the remaining 7%. Ten years ago, when the TOP500 surveys began, 27% of the listed systems used a conventional architecture, while 66% were specialized vector machines. The remaining 7% were variations on the massively parallel Connection Machine (*5*), a now-extinct species.

For some computations, which are vast in extent but simple in organization, a new computing resource with lower costs is being tapped. "Embarrassingly parallel" computations are increasingly computed at the "edge of the network." A computation is distributed to many cooperating workstations, which perform their pieces of the work with little or no coordination as background tasks. They return the completed result to a central host, which checks the results, assigns new tasks, and compiles the partial results. This approach has been used, for example, for factoring extremely large numbers and for folding a protein. But the best known example is the SETI@home project (*6*), in which over 4 million users have analyzed data from a radio telescope, seeking evidence of narrowband transmissions at likely communications frequencies. The work is done at the lowest possible priority—it is a screen saver.

The SETI@home project estimates that the total cpu power of the thousands of users active on any given day is 13 teraflops, greater than the combined power of the world's three biggest supercomputers, as listed in the 2001 TOP500 survey (*4*). However, the coordination involved in distributing and collecting all the work for the SETI@home project has consumed a significant fraction of the communications bandwidth to the outside world from the University of California's Berkeley campus.

Efforts are under way to make approaches like this applicable to a much wider class of tasks. The GRID consortium (*7*) of open-source developers is creating an infrastructure that permits jobs to be submitted anywhere within a "power grid" of reasonably reliable computer servers, under control mechanisms that will permit costs and resources to be allocated fairly. In the GRID

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